

# microRNA profiling in duodenal ulcer disease caused by *Helicobacter pylori* infection in a Western population

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## Abstract

Although the connection of microRNAs (miRNAs) to some diseases is well established, their involvement in chronic infections such as *Helicobacter pylori* has received less attention. The aim was to compare miRNA expression profiling in patients with duodenal ulcer (DU) due to *H. pylori* infection with that in infected patients without DU and in uninfected patients. The miRNA expression profile was determined by microarrays in antral mucosal samples from well-characterized dyspeptic patients ( $n = 46$ ). The most significant set of miRNAs was subsequently analysed in an independent validation group of patients ( $n = 42$ ). Transcripts for IL8, IL12p40, IL12p35 and IL23p19, the signalling molecules MYD88, GATA6, SOCS2 and STAT6 and *H. pylori* virulence factors *cagA* and *VacA* were analysed. Microarray experiments showed that 17 miRNAs were deregulated in the mucosa of *H. pylori*-infected patients. No significant differences were observed between normal and DU patients. PCR confirmed the up-regulation of *miR-9*, *miR-146a*, *miR-155* and *miR-650* and the down-regulation of *miR-96* and *miR-204* in the independent validation set of patients. Importantly, *miR-9*, *miR-96*, *miR-146a* and *miR-650* expression was specific to chronic-active gastritis. *H. pylori*-infected patients showed higher levels of IL8 and IL12p40 mRNAs and lower levels of GATA6 and SOCS2 mRNAs. The antral mucosa of patients with non-active or chronic-active gastritis showed significantly lower levels of GATA6, MYD88, SOCS2 and STAT6 mRNAs compared with patients without gastritis. The down-regulation of these factors was not correlated with the expression of any of the validated miRNAs. The exact role of the miRNA changes observed will require further study.

**Keywords:** Chronic active gastritis, duodenal ulcer disease, *Helicobacter pylori*, microRNA

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## Introduction

*Helicobacter pylori* has infected human beings for the past 58 000 years [1]. In most infected people, the bacterium acts

as a commensal organism inducing asymptomatic chronic active gastritis that can last for life. In other cases, however, it is responsible for a heavy toll of morbidity and mortality as a consequence of peptic ulcers and gastric cancer.

It is a well-known fact that *H. pylori*-infected patients with duodenal ulcer disease are at a lower risk of developing gastric cancer than patients with gastric ulcerations [2]. Chronic active gastritis in duodenal ulcer disease does not usually lead to preneoplastic changes such as atrophy or metaplasia, as it does in patients with gastric ulcer or non-ulcer dyspepsia. This wide individual response to infection has been

attributed to environmental, host and bacterial virulence factors [3]. Despite intensive research in this field, clear risk or disease-specific markers remain elusive.

Until recently, most of the known non-coding RNAs (ncRNAs) have been implicated in housekeeping functions in cells [4]. But in the past few years, thousands of genes that produce ncRNA transcripts with no recognizable open reading frame have been discovered [4,5]. MicroRNAs (miRNAs) have received considerable attention because of their impact on transcription output. miRNAs are a family of ncRNAs involved in key biological processes such as differentiation, development and cell growth [6].

miRNAs were first discovered in genetic screens of *Caenorhabditis elegans*, and play a relevant role in the development and growth of the worm [7]. This seminal discovery led to the identification of the orthologous miRNA let-7 in human cancer. Since then, numerous studies have focused on the profiling of miRNAs in this setting [8]. Studies that analyse gastric cancer have shown an altered profile of expression (reviewed in [9,10]). Although the connection of miRNAs to some diseases is well established, their involvement in the response to bacterial infectious diseases has received much less attention. In fact, only two studies have determined the miRNA expression profile in the gastric mucosa of non-cancer patients infected with *H. pylori* [11,12]. Both studies were performed in Asian populations and showed the deregulation of miRNAs in *H. pylori*-infected patients.

The aim of this study was to identify and assess the microRNA expression pattern in *H. pylori*-infected patients with duodenal ulcer disease.

## Patients and Methods

Additional information about the reference standard, *H. pylori* genotyping, PCR primers and molecular analyses can be found in Supporting Information.

### Patients

Outpatients sent to the Hospital de Sabadell for dyspeptic symptoms from October 2006 to March 2010 were prospectively recruited. Patients were contacted prior to the endoscopy and were asked to participate. Before the endoscopy, the patient signed the informed consent document in accordance with the policy and guidelines of the Ethical Committee of our institution. Those who accepted were instructed to avoid anti-secretory drugs in the 2 weeks before the test. Patients unable to stop anti-secretory drugs, those who had received antibiotics in the 4 weeks before the endoscopy and those with previous *H. pylori* treatment were excluded. During endoscopy,

antral biopsies were obtained for histology, rapid urease test (RUT) and molecular studies. As gastric biopsy specimens can show different levels of metaplasia or atrophy and miRNA profiling is capable of identifying cell lineages and tissues [13,14], patients with metaplasia or atrophy were excluded in order to avoid potential confounding variables. Reference standard-negative patients with lymphoid aggregates were also excluded.

The study design is depicted in Fig. 1 and patients' clinical and demographic data are shown in Table 1.

### Reference standard

Stringent patient selection criteria were used. A patient was classified as being *H. pylori* positive if the results of the UBT, RUT, *H. pylori* culture and histology were all positive. Patients were defined as *H. pylori* negative if all tests were negative.

### *H. pylori* genotyping

PCR was used to determine *H. pylori* *cagA* status and to genotype the *vacA* gene (signal s1/s2, intermediate i1/i2 and midregion m1/m2 polymorphisms) as described previously (detailed information on primer sequences and PCR conditions is presented in Table S1). Isolates were classified as high virulence (*cagA*+ *VacA* s1m1i1), low virulence (*cagA*- *VacA* s2m2i2) or mixed.

### High-throughput miRNA microarrays

miRNA profiling was performed with Human v1 MicroRNA expression beadchips (Illumina Inc., San Diego, CA, USA).

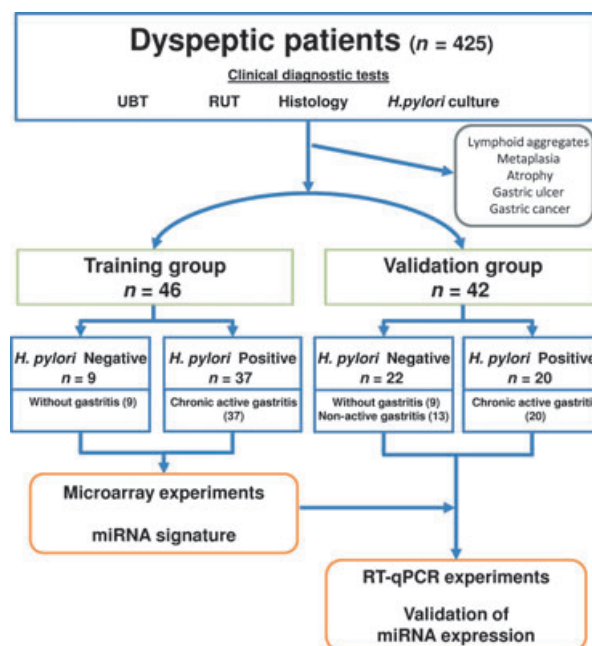


FIG. 1. Study flow diagram.

**TABLE 1.** Demographic and clinical features of the dyspeptic patients in the training and validation sets

	Training group		Validation group	
<i>H. pylori</i> status (reference standard)	Negative	Positive	Negative	Positive
<i>N</i>	9	37	22	20
Age (years)	46 ± 16.6	46 ± 14.4	47 ± 16.9	46 ± 13.9
Sex (male/female)	5/4	18/19	8/14	12/8
Endoscopic diagnosis <i>N</i> (%)				
Normal	3 (33%)	17 (46%)	8 (36%)	4 (20%)
Duodenal ulcer/erosive duodenitis	0	17 (46%)	0	9 (45%)
Oesophagitis	5 (56%)	0	7 (32%)	1 (5%)
Hiatus hernia	1 (11%)	3 (8%)	4 (18%)	2 (10%)
Other	0	0	3 (14%)	4 (20%)

### Reverse transcription and real-time PCR

Transcripts for interleukins IL8, IL23p19, IL12p35 and IL12p40 and selected signalling molecules GATA6, MYD88, SOCS2 and STAT6 were investigated (Table S2).

*miR-9*, *miR-96*, *miR-98*, *miR-137*, *miR-146a*, *miR-155*, *miR-196a*, *miR-204*, *miR-519e* and *miR-650* expression was assessed by miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark) (Table S3).

### Data and statistical analysis

The Illumina Beadchip raw data were imported to BeadStudio v3.2 (Illumina Inc.) and subsequently analysed as detailed in Supplementary Methods. LinRegPCR v12.0 was used to calculate the starting concentration per sample ( $N_0$ ) [15]. Detailed information is provided in Supporting Information.

Differences between groups were analysed using the Mann–Whitney *U*-test or the Kruskal–Wallis *H*-test as appropriate. Correlation was assessed by Spearman's rho. ROC curves were used to examine the diagnostic accuracy of miRNAs. Statistical analyses were performed with SPSS v19 (IBM Corporation, Armonk, NY, USA). Significance was set at  $p < 0.05$ .

## Results

### *H. pylori* infection alters miRNA expression in the antral mucosa

Seventeen miRNAs were deregulated in the mucosa of *H. pylori*-infected patients ( $p < 0.05$ ) (Table 2 and Fig. S1). *miR-96* was 1.5-fold down-regulated in patients with gastritis ( $p = 0.0144$ ). Globaltest analyses (Table S4) showed no significant differences with respect to sex, age, endoscopic diagnosis (normal vs. duodenal ulcer) or *H. pylori* virulence factors (high virulence (*cagA*+ *VacA* s1m1l) vs. low virulence (*cagA*- *VacA* s2m2i2)).

**TABLE 2.** Fold changes in miRNA expression as a result of infection with *H. pylori*

	Mean fold change	<i>P</i> <sup>a</sup>
Non-infected vs. infected		
Up-regulated		
hsa-miR-650	14.02	4.46E-09
hsa-miR-9*	4.32	2.36E-10
hsa-miR-9	2.79	1.29E-05
hsa-miR-519e	2.36	7.44E-13
hsa-miR-618	2.25	1.64E-05
hsa-miR-155	2.24	4.17E-13
hsa-miR-196a	2.08	8.65E-03
hsa-miR-801:9.1 <sup>b</sup>	1.91	4.64E-03
hsa-miR-766	1.87	1.29E-05
hsa-miR-146a	1.69	7.44E-13
hsa-miR-566	1.67	3.14E-02
hsa-miR-671:9.1	1.54	2.04E-02
hsa-miR-146b-5p	1.53	5.41E-11
Down-regulated		
hsa-miR-137	-1.56	1.20E-02
hsa-miR-190	-1.62	1.95E-06
hsa-miR-204	-1.64	3.01E-03
hsa-miR-502-5p	-1.52	4.54E-03
Without gastritis vs. gastritis		
Down-regulated		
hsa-miR-96	-1.50	1.45E-02

<sup>a</sup>After False Discovery Rate correction for multiple testing.

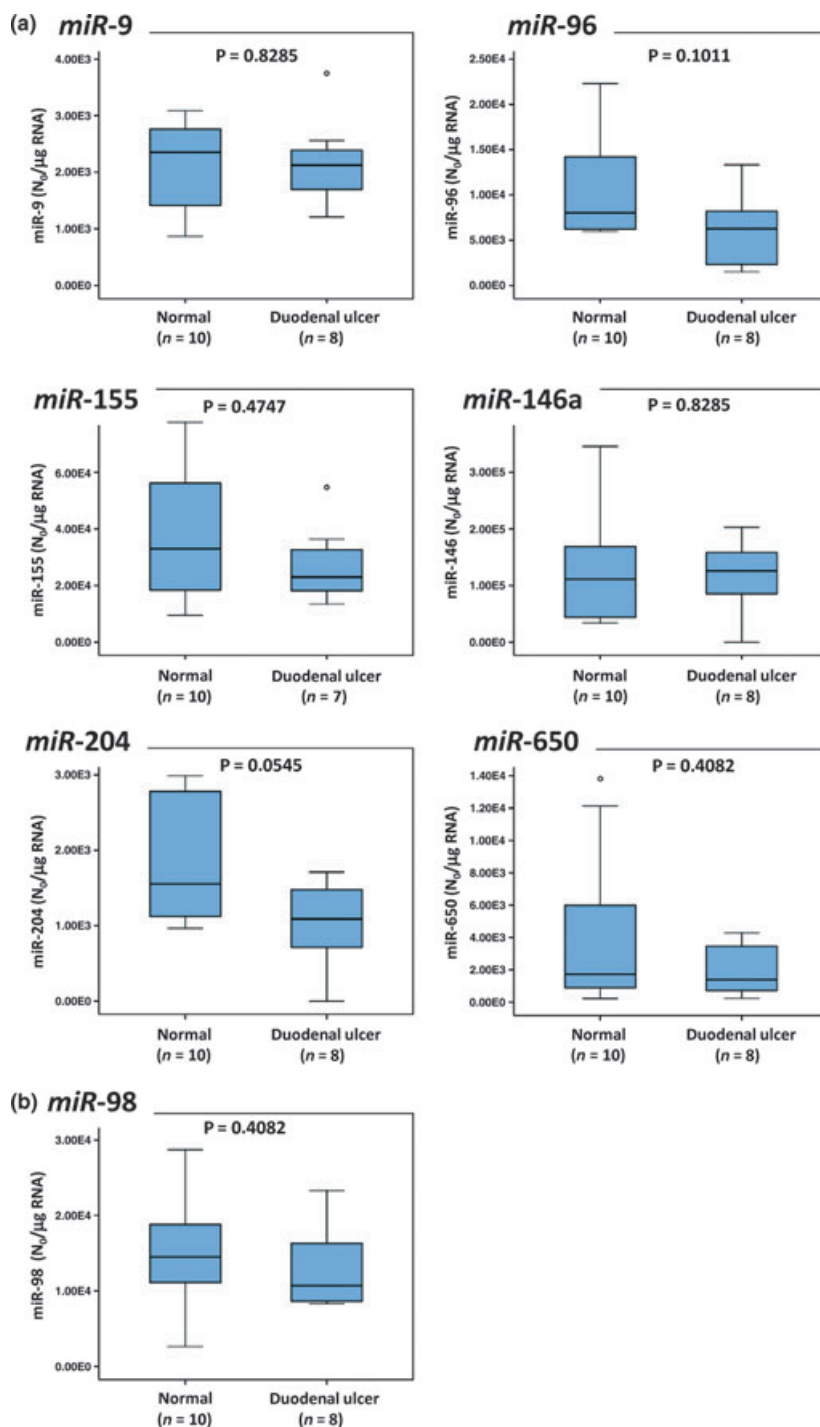
<sup>b</sup>hsa-miR-801 was removed from miRBase because it appeared to be a fragment of U11 spliceosomal RNA.

Microarray data have been deposited in NCBI's Gene Expression Omnibus [16] (GEO Series accession number GSE32174).

### The expression of the selected miRNAs is confirmed in the validation group of dyspeptic patients

miRNA RT-qPCR experiments performed on antral biopsies from the independent validation group of dyspeptic patients showed no statistical differences between normal and duodenal ulcer patients (Fig. 2). However, the increased expression of *miR-9*, *miR-146a*, *miR-155* and *miR-650* (Fig. 3a) and the down-regulation of *miR-96* and *miR-204* (Fig. 3b) was confirmed.

The highest fold increases in miRNA expression related to chronic active gastritis were for *miR-650* (c. 10-fold), followed by *miR-146a* (c. 6-fold) and *miR-155* (c. 3-fold). *miR-9*,

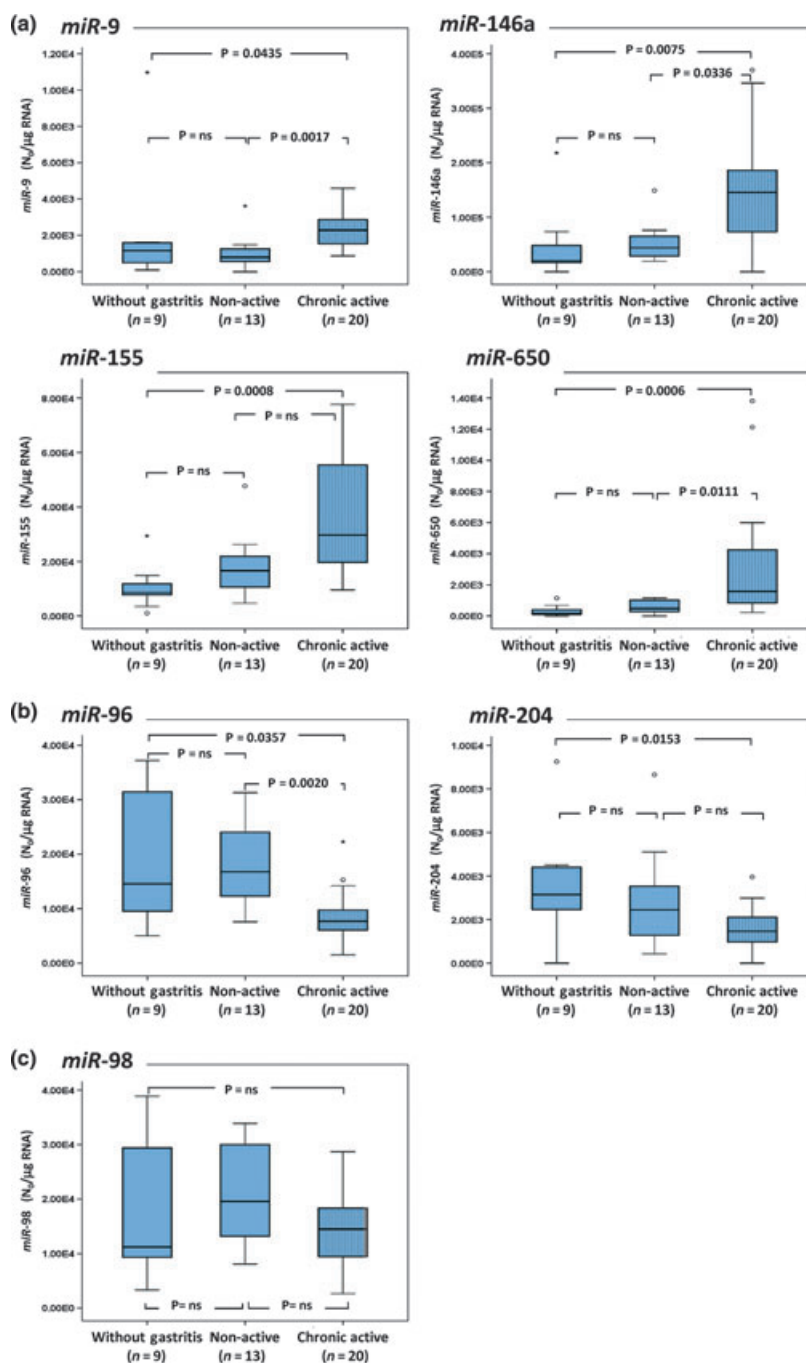


**FIG. 2.** Expression profile of the selected miRNAs in *H. pylori*-infected patients according to endoscopic findings. (a) *miR-9*, *miR-96*, *miR-146a*, *miR-155*, *miR-204* and *miR-650* expression was not different between normal and duodenal ulcer patients. (b) Expression of the 'housekeeping' microRNA *miR-98*. Mann–Whitney *U*-test. Box plots show the 25th, 50th (median), 75th and range (whiskers).

*miR-204* and *miR-96* fold-changes in chronic active gastritis vs. non-infected patients were 1.9, –2.0 and –2.4, respectively.

Non-active gastritis in the antrum is a common histological diagnosis in dyspeptic patients. In our consecutive cohort of dyspeptic patients ( $n = 425$ ), 175 patients (41%) were *H. pylori* negative. Among the *H. pylori*-negative cohort, 77% of biopsies showed mild non-active gastritis according to the

pathologist's report (unpublished observations). As non-active gastritis is very common and this group was not analysed in the microarray experiments, we decided to analyse a group of 13 additional patients who were *H. pylori* negative with non-active gastritis. RT-qPCR experiments showed no differences in miRNA expression between *H. pylori*-negative patients with and without non-active gastritis (Fig. 3a,b).



**FIG. 3.** Expression profile of the selected miRNAs in dyspeptic patients according to gastritis activity. (a) *miR-9*, *miR-146a*, *miR-155* and *miR-650* are up-regulated in patients with chronic active gastritis but not in patients with non-active gastritis. (b) *miR-96* and *miR-204* are down-regulated in patients with chronic active gastritis but not in patients with non-active gastritis. (c) Gastritis activity has no effect on the 'housekeeping' *miR-98*. Kruskal-Wallis H-test. Box plots show the 25th, 50th (median), 75th and range (whiskers).

#### Correlations for the validated miRNAs in relation to reference standard and gastritis activity

*miR-146a* and *miR-155* expressions were highly correlated (Fig. 4) and independent of *H. pylori* status (reference negative without gastritis,  $r_s = 0.9000$ ,  $p < 0.01$ ) or gastritis activity (non-active gastritis,  $r_s = 0.7032$ ,  $p < 0.01$ ; chronic active gastritis,  $r_s = 0.7108$ ,  $p < 0.01$ ). Interesting results were also found for the pair *miR-155* and *miR-204*, which were corre-

lated in infected but not in non-infected patients ( $r_s = 0.6544$ ,  $p < 0.01$ ) and in patients with active but not in patients with non-active gastritis. *miR-650* was correlated with *miR-146a* and *miR-155* in non-infected patients (*miR-650* vs. *miR-146a*,  $r_s = 0.8786$ ,  $p < 0.01$ ; *miR-650* vs. *miR-155*,  $r_s = 0.8284$ ,  $p < 0.01$ ). Table S5 summarizes the coefficients of correlations for all validated miRNAs in relation to gastritis activity.



### IL8, IL12p40, IL12p35 and IL23p19 mRNA expression

We decided to determine IL8 expression because two of the validated miRNAs from this study (*miR-155* and *miR-146a*) have been previously involved in its regulation [17–20]. Additionally, *miR-155* also modulates the expression of IL-23 [21]. IL-23 is a heterodimer formed by IL23p19 and IL12p40 subunits. The IL12p40 subunit is also shared to form the cytokine IL-12 by combining with IL12p35.

Antral biopsies from *H. pylori*-infected patients showed increased IL8 and IL12p40 mRNA expression (reference negative vs. positive: IL8,  $p < 0.00001$ ; IL12p40,  $p < 0.0001$ ) (Fig. S3), which was significantly associated with gastritis activity (non-active vs. chronic active gastritis: IL8,  $p < 0.0001$ ; IL12p40,  $p = 0.0018$ ) (Fig. 5a). Patients with duodenal ulcer or erosive duodenitis had a higher expression of IL8 mRNA than infected non-ulcer subjects ( $p = 0.0076$ ).

*In vivo* IL8, IL12p40, IL12p35 and IL23p19 expression was not correlated with the validated miRNAs (Table S6).

### Expression of the signalling molecules MYD88, GATA6, SOCS2 and STAT6

We determined MYD88 mRNA expression in our group of *H. pylori*-infected patients because *miR-9*, *miR-155* and *miR-146a* are induced by Toll-like receptor-2 (TLR-2) and TLR-2 signals through MYD88 [18]. MYD88 mRNA expression was not deregulated in *H. pylori*-infected patients (Fig. S3), and *miR-9*, *miR-155* and *miR-146a* expression was not correlated with MYD88 expression (Table S6).

Previous microarray and immunochemistry experiments [22] have revealed the mRNA and protein down-regulation of the transcription factors GATA6 and STAT6 and the suppressor of cytokine signaling-2 (SOCS2) in the antral mucosa of *H. pylori*-infected patients. We therefore sought to correlate these findings with our miRNA signature. Our cohort of patients confirms that the expression of GATA6 and SOCS2 is down-regulated in *H. pylori*-positive patients (reference negative vs. positive: GATA6,  $p = 0.0363$ ; SOCS2,  $p = 0.0060$ ) (Fig. S3). None of these genes, however, was associated with gastritis activity (Fig. 5b) or the presence of duodenal disease (not shown). GATA6, STAT6 or SOCS2 levels were not correlated with the validated miRNAs (Table S6).

### *H. pylori* virulence factors were associated with IL8 and IL12p40 mRNA levels but not with validated miRNAs

*CagA* was present in 14 (70%) out of the 20 *H. pylori* isolates. IL8 and IL12p40 mRNA levels were higher in the antral biopsies of patients infected with *cagA*<sup>+</sup> strains (IL8,  $p = 0.0340$ ; IL12p40,  $p = 0.0262$ ) (Fig. S4). Confirming our microarray results, *miR-9*, *miR-96*, *miR-98*, *miR-146a*, *miR-155*, *miR-204* and *miR-650* expression in the *H. pylori*-infected patients was

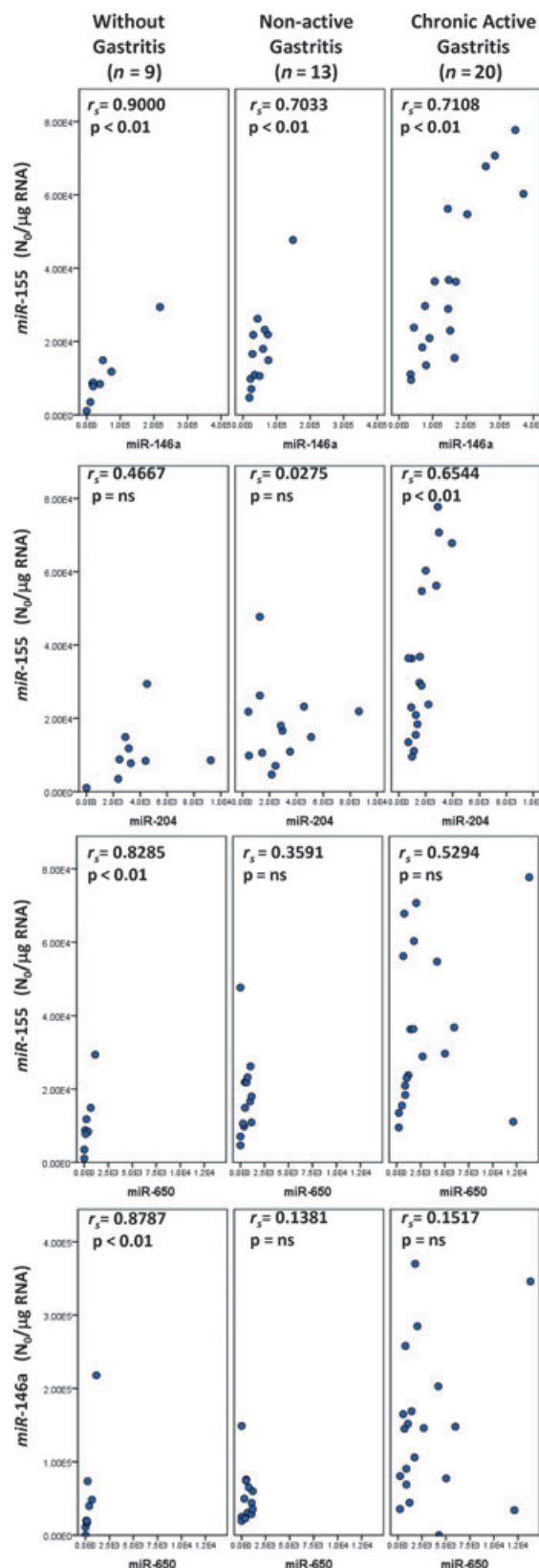
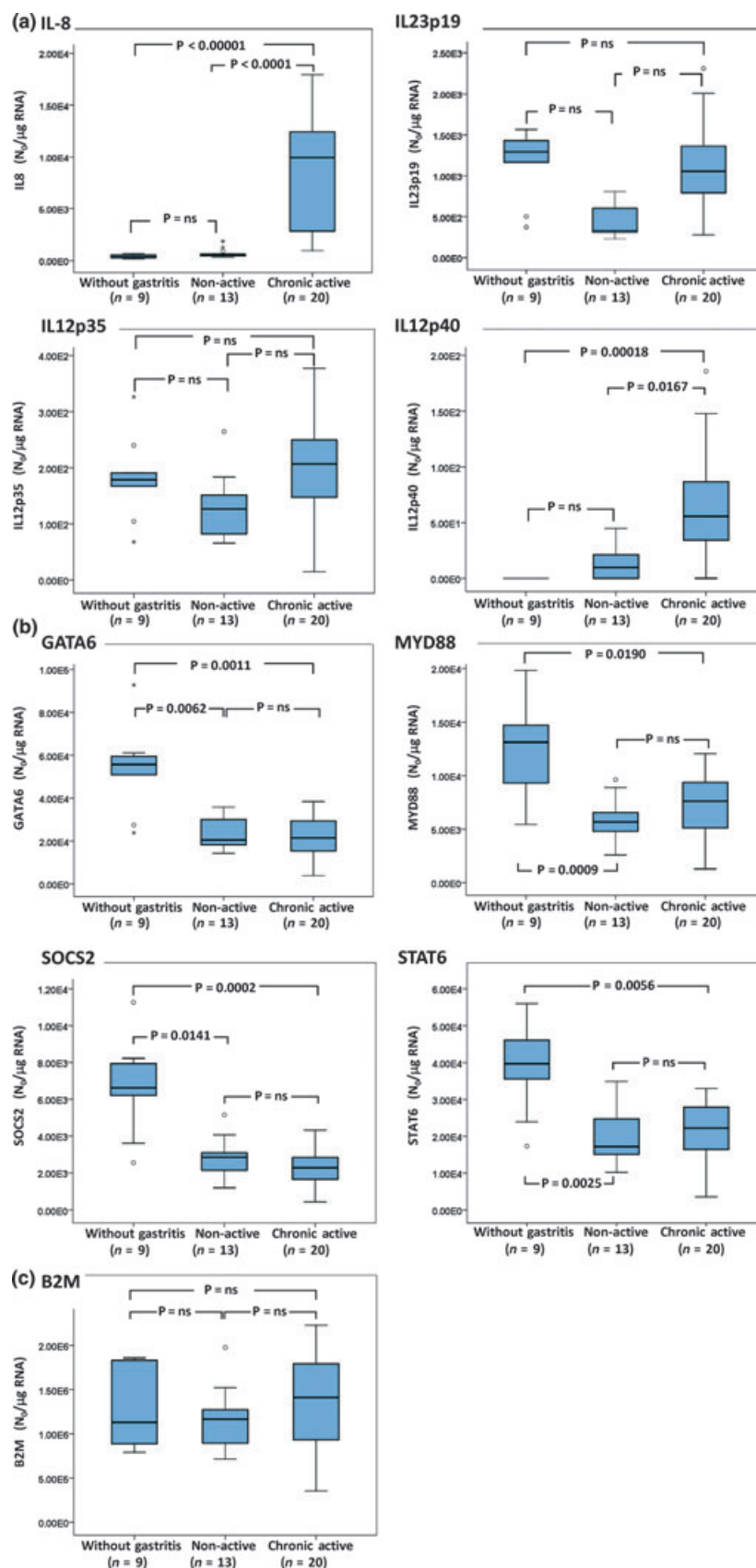


FIG. 4. Correlation plots for the selected miRNAs in relation to gastritis activity.  $r_s$ , Spearman's correlation coefficient.



**FIG. 5.** mRNA gene expression of selected interleukins (a), signalling molecules (b) and the housekeeping gene (c) in relation to gastritis activity. Kruskal-Wallis H-test. Box plots show the 25th, 50th (median), 75th and range (whiskers).

independent of the presence of *cagA* or *vacA* genotype (Table S7).

#### The ratio *miR-96/miR-155* shows the best diagnostic performance

Among the individual miRNAs, only *miR-650* showed high performance with an AUC of 0.8659 ( $p < 0.0001$ ) (Table S8). Sensitivities and specificities were improved when the ratio between down-regulated (*miR-204* and *miR-96*) and up-regulated (*miR-9*, *miR-146a*, *miR-155* and *miR-650*) miRNAs was used. Among these, the ratio *miR-96/miR-155* was the best with AUC = 0.9665 ( $p < 0.0001$ ) with a sensitivity of 84.2% and a specificity of 95.5%.

## Discussion

The present study was designed to analyse miRNA expression in human *H. pylori*-infected patients with or without duodenal ulcer disease, but with no histological diagnosis of metaplasia or atrophy. This strict patient selection criterion simplified microarray data analysis and interpretation, because miRNA signatures are characteristic of cell lineages and tissues [13]. Microarray results showed that miRNAs are differentially expressed in the *H. pylori*-infected antrum. In particular, 17 miRNAs were up-regulated and three were down-regulated in the mucosa of *H. pylori*-infected patients. The altered miRNA expression was not significantly associated with duodenal ulcer disease or with the presence of *H. pylori* virulence factors *cagA* or *vacA*. We could confirm the up-regulation of *miR-9*, *miR-146a*, *miR-155* and *miR-650* and the down-regulation of *miR-96* and *miR-204* in the independent set of dyspeptic patients.

Thus far, only one report has described the miRNA profile of *H. pylori* infection in non-cancer patients [11]. Our miRNA signature is, however, significantly different. The only factor shared by both studies is the down-regulation of *miR-204*. Several explanations can be proposed for this discrepancy. First, the population studied: the previous study was performed in Japanese patients, whereas the present study comes from a Western population. Apart from the different genetic background of the populations analysed, it is widely accepted that *H. pylori* East Asian strains are more virulent than Western ones [23]. Second, our study reports the miRNA profiling of infected patients with strict criteria for assuming the absence of infection and the presence of normal mucosa (i.e. without metaplasia or atrophy). Finally, there are some methodological differences in the microarray platforms used [24]. A major one is the preamplification step performed prior to hybridization in our study. Nevertheless,

both studies were able to confirm their respective microarray results by RT-qPCR in a validation set of patients.

TLRs play an important role in maintaining mucosal homeostasis [25]. After pathogen recognition, TLRs signal through the adaptor molecule MYD88, which activates downstream factors to coordinate the up-regulation of several functionally distinct gene subsets. Moreover, MYD88-deficient dendritic cells show impaired ability to mount local gastric inflammatory responses after *H. pylori* infection [26]. *miR-9*, *miR-146a* and *miR-155* are miRNAs regulated by TLR in different cell types and some participate in the fine-tune control of TLR signalling components [18]. *miR-155* is induced in cultured human T cells infected with *H. pylori* or treated with the purified *H. pylori* components lipopolysaccharide, VacA and  $\gamma$ -glutamyl transpeptidase through the activation of the transcription factor Foxp3 and cAMP [27]. We analysed MYD88 mRNA expression, and found no association with *H. pylori* infection. This effect may be explained by the recent observation that although MYD88 is a target of *miR-155*, it is repressed by translational repression and not by mRNA cleavage [20]. Further investigations will determine whether this apparent discrepancy reflects a different regulation of MYD88 by *miR-155* *in vivo* (gastric mucosa) or *in vitro* (cultured gastric cells). The same study reported *in vitro* inhibition of IL8 by *miR-155*. In macrophages, *miR-155* is a common target of a broad range of inflammatory mediators including MYD88 and TRIF through TLR signalling and TNF- $\alpha$  [28]. Additionally, *miR-155* shows oncogenic properties [29]. The role of *miR-155* in the induction and maintenance of chronic active gastritis provoked by *H. pylori* and its progression to intestinal metaplasia, dysplasia and eventually gastric cancer will be studied in the future.

Experiments performed with an acute monocytic leukaemia cell line stimulated with LPS have revealed that *miR-146a* expression is induced by the NF- $\kappa$ B cascade activated after the pathogen component has been recognized by the TLR system [30]. Gastric epithelial cells infected with *H. pylori* have shown that induced *miR-146a* down-regulates IRAK1 and TRAF6 proteins, participating in a negative loop that restrains the excessive activity of the pathway [31]. Others, by analysing epithelial cells and neutrophils isolated from cystic fibrosis patients, have found that *miR-146a* is also implicated in the regulation of IL8 [17]. IL-8 is a potent neutrophil-activating chemokine released by gastric epithelial cells infected with *H. pylori* [3]. The *H. pylori*-infected antral biopsies from the present study showed increased IL8 and IL12p40 mRNA expression. However, the set of validated miRNAs did not correlate with IL8 or IL12p40 expression. The cellular heterogeneity within antral biopsies may help explain the lack of *in vivo* correlation between IL8 and



miR-155 or miR-146a expression, because miRNA expression is tightly regulated within cellular populations [13,14].

More striking was the up-regulation of miR-650 in our dataset. To the best of our knowledge, miR-650 expression has not been associated with epithelial physiology or immunological response. The normal antral mucosa expressed miR-650 at low levels and it was induced in infected patients with chronic active gastritis. Very little is known about this miRNA: it has been implicated in the development of distant metastasis in gastric cancer patients [32] and is induced in tumoural samples from colorectal cancer patients [33].

Previous microarray and immunochemistry experiments [22] have demonstrated the mRNA and protein down-regulation of GATA6, STAT6 and SOCS2 in the antral mucosa of *H. pylori*-infected patients. Our results confirm the down-regulation of GATA6 and SOCS2 expression in *H. pylori* infection but we could not find a relationship between the validated miRNAs and the mRNA levels of these genes.

*H. pylori* strains harbouring multiple virulence factors (cagA+, VacA s1ml1) are associated with increased gastric mucosal inflammatory cell infiltration and increased gastric epithelial injury [34]. Accordingly, our results show the up-regulation of IL8 and IL12p40 in the antral biopsies of patients infected with cagA+ strains but we were unable to associate virulence factors with other variables. These results might be limited by sample size; therefore, additional studies will be required.

Finally, we examined the possibility of using the validated miRNAs as novel surrogate markers for *H. pylori* infection. The individual miRNA diagnostic performance was suboptimal, but by calculating the ratios between down-regulated and up-regulated miRNAs the sensitivities and specificities improved significantly, with sensitivities of c. 85% and specificities of c. 95%. The prospect that miRNAs might be used as novel surrogate markers for *H. pylori* infection opens up new possibilities for the early diagnosis of *H. pylori* infection, especially in cases in which *H. pylori* detection is difficult, for instance in patients with bleeding peptic ulcers [35].

The miRNA regulation reported by our study generates a tentative miRNA signature for *H. pylori* infection, but in order to obtain a definitive set of markers, we need to identify new, currently unknown miRNAs and demonstrate their association with infectious diseases. Microarrays are high-throughput and highly reproducible, but rely on the existing knowledge of the sequences to be studied. Therefore, high-throughput sequencing should be used to obtain complete descriptions of tissue micro-transcriptome regulation.

In conclusion, our results show that a set of miRNAs are deregulated during chronic gastric inflammation and that this set may be useful as a surrogate marker for determining the

presence of *H. pylori*. The exact role of the miRNA changes observed with the development of *H. pylori* complications and, specifically, with the appearance of gastric neoplasm will require further study.

## Acknowledgements

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## Transparency Declaration

The authors declare that no competing interests exist.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Unsupervised hierarchical clustering.

**Figure S2.** Expression profile of the selected miRNAs in the validation set of dyspeptic patients according to the reference standard for *H. pylori* infection.

**Figure S3.** Expression profile of the selected mRNAs in the validation set of dyspeptic patients according to the reference standard for *H. pylori* infection.

**Figure S4.** Gene expression of selected interleukins in relation to cagA status.

**Table S1.** Primers used for *H. pylori* genotyping.

**Table S2.** Primers used for RT-qPCR.

**Table S3.** miRNAs analyzed by RT-qPCR.

**Table S4.** Results of globaltest analysis.

**Table S5.** Spearman's correlation coefficients of miRNA expression.

**Table S6.** Correlation between miRNAs and interleukins or signaling molecules in relation to *H. pylori* status and gastritis activity.

**Table S7.** Expression of validated miRNAs in relation to *cagA* status and *vacA* genotypes.

**Table S8.** Diagnostic accuracy of IL-8 mRNA levels and selected miRNAs.

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## References

- Linz B, Balloux F, Moodley Y *et al.* An african origin for the intimate association between humans and *Helicobacter pylori*. *Nature* 2007; 445: 915–918.
- Hansson LE, Nyren O, Hsing AW *et al.* The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *N Engl J Med* 1996; 335: 242–249.
- Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. *J Clin Invest* 2007; 117: 60–69.
- Eddy SR. Non-coding RNA genes and the modern RNA world. *Nat Rev Genet* 2001; 2: 919–929.
- Matera AG, Terns RM, Terns MP. Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev Mol Cell Biol* 2007; 8: 209–220.
- Ambros V. The functions of animal microRNAs. *Nature* 2004; 431: 350–355.
- Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 1993; 75: 843–854.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; 6: 857–866.
- Wu WK, Lee CW, Cho CH *et al.* MicroRNA dysregulation in gastric cancer: a new player enters the game. *Oncogene* 2010; 29: 5761–5771.
- Belair C, Darfeuille F, Staedel C. *Helicobacter pylori* and gastric cancer: possible role of microRNAs in this intimate relationship. *Clin Microbiol Infect* 2009; 15: 806–812.
- Matsushima K, Isomoto H, Inoue N *et al.* MicroRNA signatures in *Helicobacter pylori*-infected gastric mucosa. *Int J Cancer* 2011; 128: 361–370.
- Xiao B, Liu Z, Li BS *et al.* Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. *J Infect Dis* 2009; 200: 916–925.
- Landgraf P, Rusu M, Sheridan R *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007; 129: 1401–1414.
- Petriv OI, Kuchenbauer F, Delaney AD *et al.* Comprehensive microRNA expression profiling of the hematopoietic hierarchy. *Proc Natl Acad Sci USA* 2010; 107: 15443–15448.
- Ruijter JM, Ramakers C, Hoogaars WM *et al.* Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 2009; 37: e45.
- Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 2002; 30: 207–210.
- Bhattacharyya S, Balakathiresan NS, Dalgard C *et al.* Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyperexpression of interleukin-8. *J Biol Chem* 2011; 286: 11604–11615.
- O'Neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of toll-like receptor signalling. *Nat Rev Immunol* 2011; 11: 163–175.
- Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1 $\beta$ -induced inflammatory response in human lung alveolar epithelial cells. *J Immunol* 2008; 180: 5689–5698.
- Tang B, Xiao B, Liu Z *et al.* Identification of MyD88 as a novel target of miR-155, involved in negative regulation of *Helicobacter pylori*-induced inflammation. *FEBS Lett* 2010; 584: 1481–1486.
- Ceppi M, Pereira PM, Dunand-Sauthier I *et al.* MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci USA* 2009; 106: 2735–2740.
- Hofman VJ, Moreillon C, Brest PD *et al.* Gene expression profiling in human gastric mucosa infected with *Helicobacter pylori*. *Mod Pathol* 2007; 20: 974–989.
- Suerbaum S, Josenhans C. *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nat Rev Microbiol* 2007; 5: 441–452.
- Git A, Dvinge H, Salmon-Divon M *et al.* Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA* 2010; 16: 991–1006.
- Sabroe I, Dower SK, Whyte MK. The role of toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. *Clin Infect Dis* 2005; 41 (Suppl 7): S421–S426.
- Rad R, Brenner L, Krug A *et al.* Toll-like receptor-dependent activation of antigen-presenting cells affects adaptive immunity to *Helicobacter pylori*. *Gastroenterology* 2007; 133: 150–163.
- Fassi Fehri L, Koch M, Belogolova E *et al.* *Helicobacter pylori* induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS ONE* 2010; 5: e9500.
- O'Connell RM, Kahn D, Gibson WS *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 2010; 33: 607–619.
- Lawrie CH. MicroRNA expression in lymphoma. *Expert Opin Biol Ther* 2007; 7: 1363–1374.
- Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 2006; 103: 12481–12486.
- Liu Z, Xiao B, Tang B *et al.* Up-regulated microRNA-146a negatively modulate *Helicobacter pylori*-induced inflammatory response in human gastric epithelial cells. *Microbes Infect* 2010; 12: 854–863.
- Zhang X, Zhu W, Zhang J *et al.* MicroRNA-650 targets ING4 to promote gastric cancer tumorigenicity. *Biochem Biophys Res Commun* 2010; 395: 275–280.
- Feng L, Xie Y, Zhang H, Wu Y. Down-regulation of NDRG2 gene expression in human colorectal cancer involves promoter methylation and microRNA-650. *Biochem Biophys Res Commun* 2011; 406: 534–538.
- Cover TL, Blaser MJ. *Helicobacter pylori* in health and disease. *Gastroenterology* 2009; 136: 1863–1873.
- Ramirez-Lazaro MJ, Lario S, Casals A *et al.* Real-time PCR improves *Helicobacter pylori* detection in patients with peptic ulcer bleeding. *PLoS One* 2011; 6: e20009.